(19) Europäisches Patentamt
European Patent Office
Office européen des brevets

(11) EP 3 312 275 A1

(12) EUROPEAN PATENT APPLICATION
published in accordance with Art. 153(4) EPC

(43) Date of publication:
25.04.2018 Bulletin 2018/17

(21) Application number: **16779579.8**

(22) Date of filing: **12.04.2016**

(51) Int Cl.:
C12N 9/88 *(2006.01)* ***C12N 15/60*** *(2006.01)*
A61K 38/51 *(2006.01)* ***A61P 31/04*** *(2006.01)*
A61P 1/02 *(2006.01)*

(86) International application number:
PCT/CN2016/079043

(87) International publication number:
WO 2016/165604 (20.10.2016 Gazette 2016/42)

(84) Designated Contracting States:
AL AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MK MT NL NO PL PT RO RS SE SI SK SM TR
Designated Extension States:
BA ME
Designated Validation States:
MA MD

(30) Priority: **13.04.2015 CN 201510171835**

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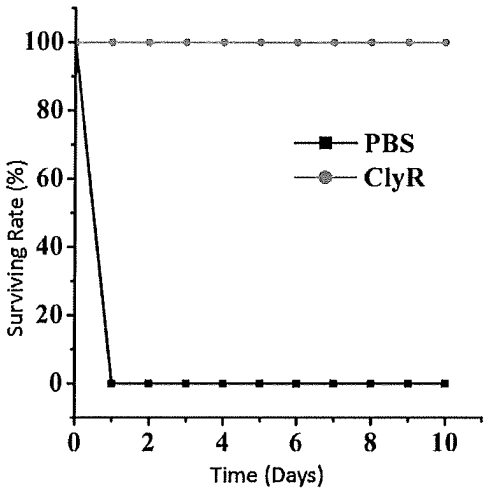
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(54) BROAD SPECTRUM OF STREPTOCOCCUS LYASE AND USE THEREOF

(57) This invention discloses a lysin that can kill many species of *Streptococci.* A new lysin, ClyR, was constructed by the gene splicing method. The ClyR can effectively kill different species of *Streptococci,* including *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus mutans, Streptococcus equi,* and various *Enterococci* and *Staphylococcus aureus.* ClyR shows good stability and is not sensitive to EDTA and high concentration of NaCl. Moreover, the ClyR is active in a wide range of pH and maintains high activity in pH 5-11. Recombinant protein ClyR is well expressed in *E. coli* stain BL21 (DE3). High doses of ClyR showed no apparent toxicity in mice. Furthermore, administration of 0.8 mg per mouse once is able to completely protect the mouse infected with lethal doses of Group B *Streptococci.* The ClyR can be used alone or in combination with different forms of reagents and solutions, for the control of a variety of *Streptococci* and for the treatment of infections caused by these bacteria. It has a broad application prospect.

Figure 7



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Description

Technical Field

[0001] The invention belongs to the microbiology field, in particular relates to a kind of lysin that is capable of killing *Streptococci, Enterococci* and *Staphylococcus aureus,* and its coding gene, and the use thereof as antimicrobial.

Background

[0002] *Streptococcus* is a class of spherical Gram-positive bacteria that cause many diseases in humans and animals. *Streptococcus pneumoniae* is the main pathogenic bacteria causing lobar pneumonia and other diseases. It more frequently causes bronchopneumonia among infants and children. *Streptococcus pyogenes* is widely distributed in nature, and is one of the most important human bacterial infection pathogens. It can cause a variety of suppurative inflammation, including scarlet fever, erysipelas, neonatal sepsis, meningitis, *Streptococcus* allergy and so on. Group B *Streptococci,* such as *Streptococcus agalactiae,* is one of the important pathogens of perinatal infection, which seriously threatens the health of the newborns. *Streptococcus. mutans* is the main pathogen of dental caries, which seriously threatens the oral health of human beings. At present, there is no effective vaccine for the prevention and treatment of dental caries. In addition, *Streptococci* can cause a number of important animal diseases and threaten animal husbandry seriously. *Streptococcus suis* disease is a zoonotic acute and febrile infectious disease, usually caused by group C, D, E, and L *Streptococci.* Mastitis, the most important disease of farming industry in dairy cows, is caused by four main pathogenic bacteria, *Streptococcus agalactiae, Streptococcus uberis, Streptococcus dysgalactiae* and *Staphylococcus aureus.* However, there is still lack of effective vaccine and treatment strategy today. From the above, we can see that *Streptococcus* species are varied and can cause a large number of diseases of human beings and animals.

[0003] Phage lysin is a type of cell wall hydrolase expressed in late stage after dsDNA phage infecting host bacteria. Usually the size of the lysin is 25 kD~40 kD. Structurally, lysin is composed of two independent functional domains, the N-terminal catalytic domain and the C-terminal cell binding domain (CBD) that determines cell binding sites. The two domains are linked by a small fragment. Sequence analysis shows that the catalytic domain of the same type of lysin is highly conserved, while the cell binding domain is variable, which provides the possibility to construct a new chimeric lysin. Lysins are highly specific and can only specifically identify and lyse specific species of bacteria. In addition, the cleavage site is very well conserved, and the specificity of the phage and bacterial coevolution makes it difficult for host bacteria to develop resistance to them. These lysins characteristics provide theoretical feasibility for the control and treatment of drug-resistant bacteria in clinical practice. Until now, a few natural lysins that acts upon *Streptococci* have been reported. These lysins can kill specific strains of *Streptococci* both *in vivo* and *in vitro.* However, these lysins usually have a narrow lytic spectrum, and most of them are difficult to be solubly expressed, or do not high active, and active in a narrow range of pH, generally in pH 5-8. At present, none of lysins reported is able to lyse *Streptococcus mutans* effectively. It is very importantly significant to look for lysins, which are soluble, able to be expressed in high level, and show broad spectrum of activity, for development of new anti-streptococcal drugs and to control of streptococcal infections *in vitro* and so on.

Disclosure of Invention

[0004] one technical problem to be solved in this invention is to provide a lysin, which is capable of killing a high variety *Streptococci.* The lysin can kill *Streptococci in vitro and in vivo,* especially *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus mutans, Streptococcus equi* and various *Enterococci* and *Staphylococcus. aureus.* For the convenience of narration, we name this lysin as ClyR, and its coding gene as *ClyR.*

[0005] The nucleic acid sequence of the coding gene *ClyR of Streptococcus* lysin provided in the present invention is shown in SEQ.ID.NO.1.

[0006] The protein sequence of the *Streptococcus* lysin ClyR provided in the present invention is shown in SEQ.ID.NO.2.

[0007] The invention also provides a method for expression of soluble ClyR protein and its purification. The steps are as follows: the *Cly**R*** gene is cloned, and then ligated with expression vector pBAD24, then expression plasmid is transferred *E.coli* BL21 (DE3) and is expressed , the expressed protein is firstly purified by ion exchange, then treated with dialytic phosphate buffer (PBS).

[0008] The invention demonstrate that the ClyR has effect on killing a large variety of *Streptococci* and *Staphylococcus aureus* via vitro experiment, especially *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus uberis, Streptococcus agalactiae, Streptococcus dysgalactiae, Streptococcus mutans, Streptococcus equi* and various *Enterococci* and *Staphylococcus aureus,* which proves the high activity and broad spectrum anti-bacterial activity

of this lysin. The invention also preliminarily tested the protective effect of ClyR in the experimental animal model mice infected with Group B *Streptococcus,* and tested the cytotoxicity, which initially confirmed the potential of the drug for the development of anti-streptococcal infections.

[0009] The invention has the following beneficial effects and advantages:

[0010] The ClyR according to the invention can be used for *in vivo and in vitro* killing of a large variety *Streptococci* and *Staphylococcus aureus.* ClyR can be expressed in *Escherichia coli* in soluble form, and has high enzyme activity and relatively high activity in the range of pH 5-11. It can kill *Streptococci* with broad spectrum, and has the potential to be applied to antimicrobial drugs *in vivo.*

Brief Description of the Drawings

[0011]

Figure 1 shows the PCR amplification of the gene *ClyR.* M is the standard molecular weight marker. The arrow points to 750 bp. R is the band of the amplified *ClyR.*

Figure 2 demonstrates the kinetics of the killing effect of ClyR on *Streptococcus dysgalactiae* standard strain ATCC 35666 over time. The solid rectangle shows the change trend of Log CFU over time after *Streptococcus dysgalactiae* being mixed with ClyR. The hollow circle shows the change trend of OD_{600} over time after *Streptococcus dysgalactiae* being mixed with buffer. The hollow triangle shows the change trend of OD_{600} over time after *Streptococcus dysgalactiae* being mixed with ClyR.

Figure 3 is the result of the effect of EDTA on ClyR activity. *Streptococcus dysgalactiae* standard strain ATCC 35666 was re-suspended in PBS buffer with different EDTA concentration, and then mixed with the same concentration of ClyR to detect the change trend of OD_{600} over time. The vertical coordinates indicate the relative activity of ClyR under different conditions.

Figure 4 is the result of the effect of pH on ClyR activity. *Streptococcus dysgalactiae* standard strain ATCC 35666 was re-suspended in the buffer with different pH, and then mixed with the same concentration of ClyR to detect the change trend of OD_{600} over time. The vertical coordinates indicate the relative activity of ClyR under different conditions.

Figure 5 shows the effect of NaCl on ClyR activity. *Streptococcus dysgalactiae* standard strain ATCC 35666 was re-suspended in the buffer with different NaCl concentration, and then mixed with the same concentration of ClyR to detect the change trend of OD_{600} over time. The vertical coordinates indicate the relative activity of ClyR under different conditions.

Figure 6 demonstrates the result of the broad spectrum of ClyR killing *Streptococci, Staphylococcus* and other bacteria *in vitro.* The vertical coordinates indicate the decreased values of OD_{600} after different strains being mixed with ClyR and incubated at 37°C for 15 min.

Figure 7 shows the result of the killing effect of ClyR on *Streptococci in vivo.* 20 mice in each group were injected with lethal doses of *Streptococcus agalactiae,* individually. After 3 hours, the mice in the experimental group were injected with 0.8 mg ClyR intraperitoneally; while the mice in the control group were injected with an equal volume of PBS solution intraperitoneally after 3 hours. The survival rate of the mice in each group was observed each day.

Figure 8 shows results of the cytotoxicity results of ClyR. the concentrations of 0.1 mg/ml, 0.5mg/ml and 1 mg/ml of ClyR are applied to Caco-2 cells respectively, no significant cytotoxicity is observed. Ionomycin and Mitomycin C which are well known to be toxic to these cells were used as positive controls.

Figure 9 demonstrate the result of the killing effect of ClyR on *Streptococcus* in milk. *Streptococcus dysgalactiae* ATCC 35666 was mixed with sterile milk and then treated with different concentrations of ClyR for 1 hour. Bacterial numbers on plates were counted after serial dilution.

Detailed Description of the Invention

[0012] The present invention is further explained by the following examples, but the present invention is not to be limited by this.

[0013] We designed and synthesised a type of new *Streptococcus* lysin designated ClyR through the analysis of amino acid sequences of the catalyst domain and the cellar structure domain of *Staphylococcus* phage lysin.

[0014] The methods used in the following examples were standard experimental methods without special description. All primers used in experiments were provided by Invitrogen (Shanghai). DNA sequencing was performed by Invitrogen (Shanghai).

Example 1

Construction of the lysin with ability of specifically killing *Streptococci*

[0015]

1) The *ClyR* gene sequence expressing lysin ClyR was totally synthesised by Genscript (Nanjing). The synthetic sequence was inserted into plasmid pUC57. The *ClyR* gene was used as a template. Restriction enzyme sites NcoI and XhoI were introduced at two terminals of the target gene. Primers were designed as below:

Forward primer:

5-TATACCATGGGCATGGCAGCAAATCTGG -3 (SEQ.ID.NO.3)
NcoI

Reverse primer:

5-ATATCTCGAGTTTGAAGGTACCCCATGCGTTG -3(SEO.1D.NO.4)
XhoI

2μl of gene was used as a template, 1μg of each primer was added for PCR amplification, and the programme of PCR amplification was described as below:

(1) 94 °C, 5 min;
(2) 94 °C, 30 sec, 62 °C, 45 sec, 72 °C, 45 sec, 30 cycles;
(3)72 °C, 10 min.

PCR products were electrophoresed and recovered, the data of electrophoresis was shown in Figure 1. The size of *ClyR* gene was 759bp, which was same as the designed lysin.

2) The *ClyR* gene was ligated with expression plasmid pBAD24 to obtain a recombinant plasmid pB-ClyR, and then this recombinant plasmid was transferred into *E.coli* strain BL21(DE3).

3) Expression and purification of ClyR

[0016] Expression strain BL21(DE3)/pB-ClyR was induced by 0.2% L-arabinose to express the lysin under low temperature. Bacteria were collected, and then sonicated, the supernatant was obtained and precipitated by 33% ammonium sulphate. The precipitate was then dissolved in PBS and dialysed against PBS overnight. The dialysate had obvious anti-bacterial activity.

[0017] The crude extract after dialysis or the supernatant after sonication was passed through a HiTrap Q Sepharose FF column (GE Healthcare), and the effluent was collected. The effluent was then passed through a HiTrap SP Sepharose FF column, and the sample was eluted by using a gradient of 1M NaCl, and the elution peaks were collected separately. The fractions with activity were combined, and then dialysed against PBS overnight. The resulting sample was a purified enzyme.

Example 2

Verification of the killing effect of ClyR on standard *Streptococcus dysgalactiae* strain ATCC 35666

[0018] An overnight culture of *Streptococcus dysgalactiae* strain ATCC 35666 was collected by centrifugation, and

washed with PBS once, and then re-suspended in PBS. A certain amount of ClyR was mixed with the above bacteria culture, at the same time, the change of absorbance at 600nm and the number of viable bacteria was monitored by a microplate reader. The mixture of buffer and *Streptococcus dysgalactiae* was used as a negative control. The final lysis curve was obtained as shown in Figure 2. This result showed that ATCC 35666 strain was rapidly lysed by ClyR, as a result, absorbance at 600nm was rapidly declined.

Example 3

Verification of the effect of EDTA on the activity of ClyR

[0019] An overnight culture of *Streptococcus dysgalactiae* strain ATCC 35666 was collected by centrifugation, and washed with PBS once, and then re-suspended in PBS. Bacteria suspensions were then divided into multiple parts, and different concentrations of EDTA were added, respectively, and then well mixed.

[0020] A certain amount of ClyR was mixed with the above bacteria culture, at the same time, the change of absorbance at 600 nm of mixture was monitored by a microplate reader. The mixture of buffer and *Streptococcus dysgalactiae* was used as a negative control. After detection, the reduced values of OD_{600} between each test group and the control group were calculated, the maximum reduced values of OD_{600} was defined as 1, the reduced values of OD_{600} in other groups were compared with said value, and then the relative enzyme activity was obtained. The final lysis curve was obtained as shown in Figure 3. The results showed that EDTA had less effect on the activity of ClyR.

Example 4

Verification of the effect of pH on the activity of ClyR

[0021] An overnight culture of *Streptococcus dysgalactiae* strain ATCC 35666 was collected by centrifugation, and washed once with PBS, and then re-suspended in buffer with different pH. A certain amount of ClyR was mixed with the above bacteria suspensions, at the same time, the change of absorbance at 600 nm of mixture was monitored by a microplate reader. The mixture of buffer and *Streptococcus dysgalactiae* was used as a negative control. After detection, the reduced values of OD_{600} between each test group and the control group were calculated, the maximum reduced values of OD_{600} was defined as 1, the reduced values of OD_{600} in other groups were compared with said value, and then the relative enzyme activity was obtained. The final lysis curve was obtained as shown in Figure 4. The results demonstrate that ClyR show good activity in pH range of 5-11.

Example 5

Verification of the effect of NaCl on the activity of ClyR

[0022] An overnight culture of *Streptococcus dysgalactiae* strain ATCC 35666 was collected by centrifugation, and washed once with PBS, and then re-suspended in PBS. Bacteria suspensions were then divided into multiple portions, different concentrations of NaCl was added, respectively, and then well mixed. A certain amount of ClyR was mixed with the above bacteria suspensions, at the same time, the change of absorbance at 600 nm of mixture was monitored by a microplate reader. At same time The mixture of buffer and *Streptococcus dysgalactiae* was used as a negative control. After detection, the reduced values of OD_{600} between each test group and the control group were calculated, the maximum reduced values of OD_{600} was defined as 1, the reduced values of OD_{600} in other groups were compared with said value, and then the relative enzyme activity was obtained. Final lysis curve was obtained as shown in Figure 5. The results showed that NaCl had less effect on the activity of ClyR.

Example 6

Verification of spectrum of anti-bacterial activity of ClyR against *Streptococcus, Staphylococci* and other bacteria strains

[0023] An overnight culture of different *Streptococcus, Staphylococcus* and other reference stains were collected by centrifugation, and washed with once PBS, and then re-suspended in PBS. A certain amount of ClyR was mixed with the above bacteria suspension, at the same time, the change of absorbance at 600nm of mixture was monitored by a microplate reader for 15 minutes. The reduced values of OD_{600} indicated the lytic effect against different strains. At the same time, the mixture of buffer and bacteria suspension was used as a negative control. The killing curve obtained from this experiment was shown in Figure 6. The result show ClyR was able to rapidly kill multiple *Streptococci* (especially *Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus uberis, Streptococcus aga-*

lactiae, Streptococcus dysgalactiae, Streptococcus mutans or Streptococcus, equi), and also *Staphylococcus,* but there was no lytic activity against other tested strains.

Example 7

Verification of the anti-Streptococcus activity of ClyR *in vivo*

[0024] approximately 20 to 22g, 6 weeks old female BALB/c mice were used in this experiment; weights of mice were. Experimental mice (40 mice) were injected intraperitoneally with 6×10^8 of *Streptococcus agalactiae* strain. After 3 hours, mice were divided into two groups (20 mice in each group). The mice in the test group were injected intraperitoneally with 800μg ClyR, and the mice in the control group were injected intraperitoneally with PBS buffer. The survival rate of the mice was observed every day, and the results were shown in Figure 7. This result showed that ClyR was able to rapidly kill *Streptococcus in vivo,* therefore, the survival rate of the mice was increased.

Example 8

Verification of the no cytotoxicity of ClyR

[0025] Caco-2 cells were inoculated into 96 well plates at a concentration of 5×10^3 cells per well. After incubation for 24 hours, a certain concentration of ClyR (0.1-1mg/ml), ionomycin (15mg/ml) and mitomycin (15mg/ml) were added into wells. After further incubation for 24 hours, the staining reagent WST-8 was added into wells. After standing, the absorbance at 450nm were measured. The obtained results were shown in Figure 8. The results showed that ClyR had no cytotoxicity in high concentration.

Example 9

Verification of the elimination of *Streptococcus* in milk using ClyR

[0026] An overnight culture of *Streptococcus* was collected by centrifugation, and washed with PBS once, and then re-suspended in sterile milk. Different concentrations of ClyR were mixed with the above bacterial suspensions, after mixed well, the reaction suspensions were incubated at 37°C for 1 hour, and then the number of viable bacteria was calculated by serial dilution and counting on plates. Experimental results were shown in Figure 9. The results showed that this enzyme was able to effectively eliminate *Streptococcus* in milk.

SEQUENCE LISTING

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Claims

1. A *Streptococcus* lysin ClyR, wherein the protein sequence of said lysin is shown in SEQ.ID.NO.2.

2. A ***ClyR*** gene coding *Streptococcus* lysin according to claim 1, wherein the DNA sequence of this gene is shown in SEQ.ID.NO.1.

3. A method of soluble expression and purification of *Streptococcus* lysin ClyR according to claim 1, **characterized in that** the ***ClyR*** gene is cloned and ligated with expression vector pBAD24, then expression plasmid transferred in *E.coli* BL21 (DE3) and is expressed; the expressed protein is firstly purified by ion exchange chromatography, and then treated with dialytic phosphate buffer.

4. Use of the *Streptococcus* lysin ClyR according to claim 1 to kill *Streptococcus, Enterococcus and Staphylococcus aureus* in animals and humuan beings *in vitro.*

5. Use according to claim 4, **characterized in that** said *Streptococci is Streptococcus pneumoniae, Streptococcus pyogenes, Streptococcus suis, Streptococcus uberis, Streptococcus agalactiae, Streptococcus. dysgalactiae, Streptococcus mutans or Streptococcus equi.*

6. Use according to claim 4, **characterized in that** the use of the *Streptococcus* lysin ClyR as described in claim 1 is killing *Streptococcus* in milk.

7. Use of the *Streptococcus* lysin ClyR according to claim 1 for the manufacturing of a medicament against Streptococcus infection.

8. Use according to claim 7, **characterized in that** the use of the *Streptococcus* lysin ClyR as described in claim 1 for the manufacturing of a medicament for the prevention and/or treatment of bovine mastitis.

9. Use according to claim 7, **characterized in that** the use of the *Streptococcus* lysin ClyR as described in claim 1 for the manufacturing of a medicament for the prevention and/or treatment of dental caries.

10. An antibiotic, containing the *Streptococcus* lysin ClyR according to claim 1.

Figure 1

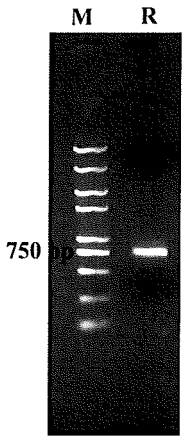


Figure 2

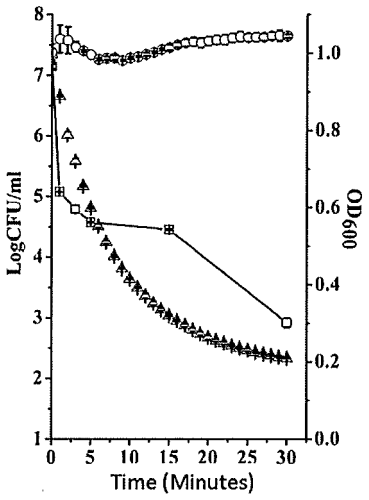


Figure 3

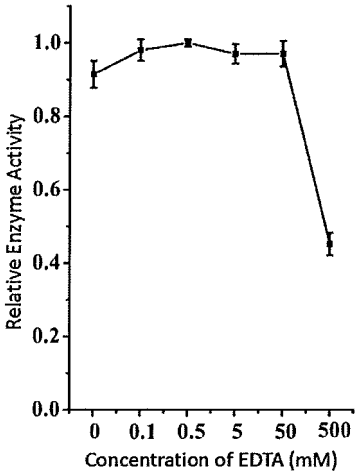


Figure 4

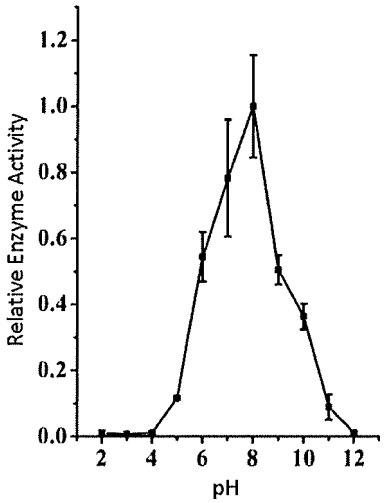


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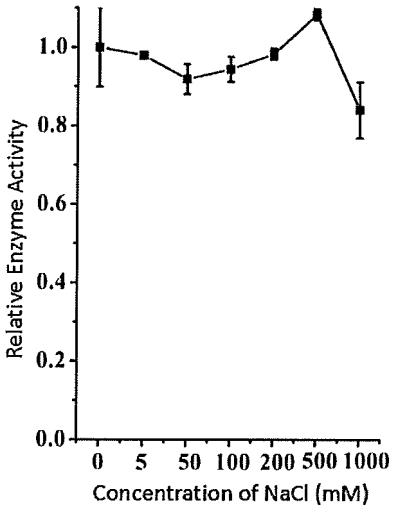

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Figure 6

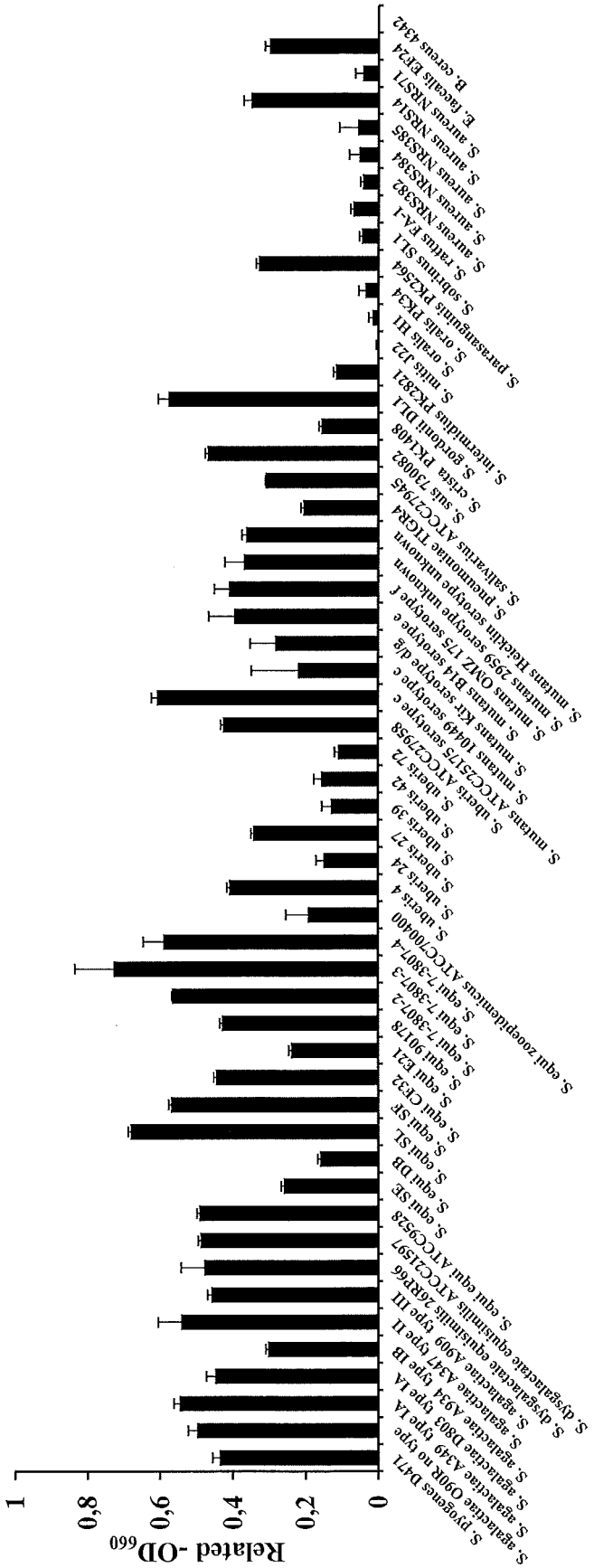

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S. pneumoniae unknown
S. salivarius ATCC27945
S. suis 730082
S. crista PK1408
S. gordonii DL1
S. intermitdius PK2821
S. mitis 122
S. oralis H1
S. parasanguinis PK2564
S. sobrinus PK2564
S. tatus EA-1
S. aureus NRS382
S. aureus NRS384
S. aureus NRS385
S. aureus NRS14
S. aureus NRS71
B. cereus 4342
B. cereus EJE24

Figure 7

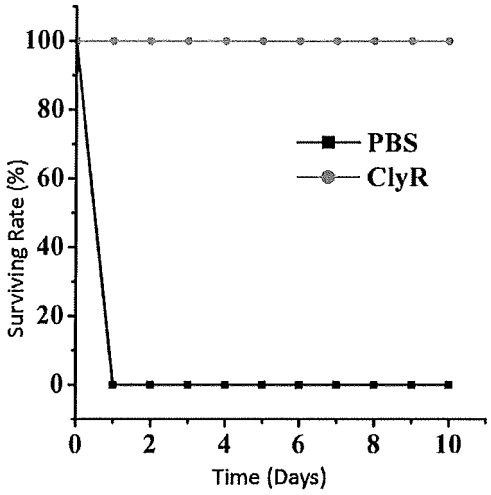


Figure 8

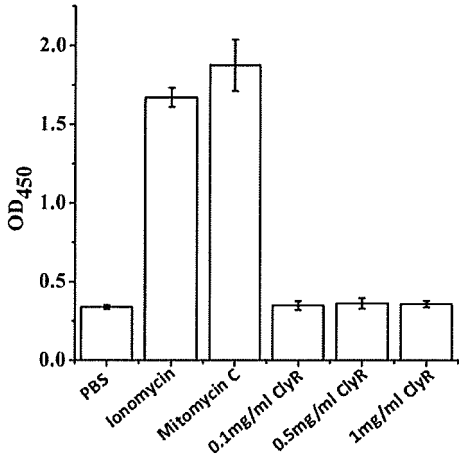
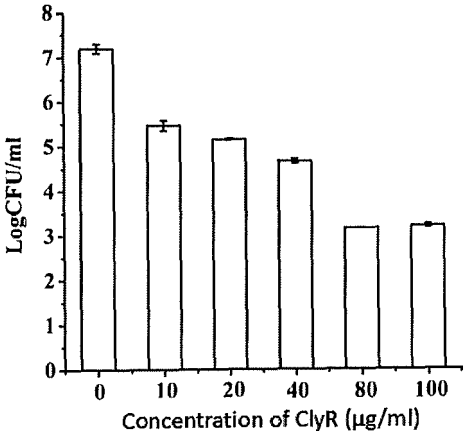


Figure 9


LogCFU/ml
0
1
2
3
4
5
6
7
8
0
10
20
40
80
100
Concentration of ClyR (μg/ml)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/CN2016/079043

A. CLASSIFICATION OF SUBJECT MATTER

C12N 9/88 (2006.01) i; C12N 15/60 (2006.01) i; A61K 38/51 (2006.01) i; A61P 31/04 (2006.01) n; A61P 1/02 (2006.01) n
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CPRS, CNABS, WPI, EPODOC, SIPOABS, CNKI, BA, PUBMED, Genbank, DDBJ, EMBL: bacteriophage lysine, sequence search on the sequences of SEQ ID NO: 1 and 2

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
PX	CN 104726439 A (WUHAN SAISIRUI MICROBIAL TECHNOLOGY CO.) 24 June 2015 (24.06.2015), see the abstract, claims 1-10	1-10
A	CN 103122347 A (WUHAN VIROLOGY ACAD INST SINICA) 29 May 2013 (29.05.2013) , see the abstract, and claims 1-6	1-10
A	CN 102181420 A (UNIV SHANGHAI JIAOTONG) 14 September 2011 (14.09.2011) see the whole document	1-10
A	CN 103443269 A (US SEC OF AGRIC) 11 December 2013 (11.12.2013) see the whole document	1-10
A	CN 103080307 A (ALERE SCARBOROUGH INC.) 01 May 2013 (01.05.2013) see the whole document	1-10

☒ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

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"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&"document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 June 2016	14 July 2016
Name and mailing address of the ISA State Intellectual Property Office of the P. R. China No. 6, Xitucheng Road, Jimenqiao Haidian District, Beijing 100088, China Facsimile No. (86-10) 62019451	Authorized officer SONG, Zhigang Telephone No. (86-10) 62411078

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INTERNATIONAL SEARCH REPORT
Information on patent family members

International application No.
PCT/CN2016/079043

Patent Documents referred in the Report	Publication Date	Patent Family	Publication Date
CN 104726439 A	24 June 2015	None	
CN 103122347 A	29 May 2013	CN 103122347 B	22 April 2015
CN 102181420 A	14 September 2011	None	
CN 103443269 A	11 December 2013	US 2012052546 A1	01 March 2012
		WO 2012030535 A2	08 March 2012
		WO 2012030535 A3	31 May 2012
		EP 2611913 A2	10 July 2012
		JP 2013539965 A	31 October 2012
		US 8962297 B2	24 February 2015
CN 103080307 A	01 May 2013	WO 2011091412 A1	28 July 2011
		EP 2539446 A1	02 January 2013
		US 2013029351 A1	31 January 2013
		US 8951532 B2	10 February 2015
		US 2015198595 A1	16 July 2015

Form PCT/ISA /210 (patent family annex) (July 2009)